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DOI:

[10.1016/j.cbpa.2017.11.002](https://doi.org/10.1016/j.cbpa.2017.11.002)

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*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Griffiths, R, Kocurek, K & Cooper, H 2018, 'Ambient surface mass spectrometry-ion mobility spectrometry of intact proteins', *Current Opinion in Chemical Biology*, vol. 42, pp. 67-75.  
<https://doi.org/10.1016/j.cbpa.2017.11.002>

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# Ambient surface mass spectrometry–ion mobility spectrometry of intact proteins

Rian L Griffiths, Klaudia I Kocurek and Helen J Cooper



Ambient surface mass spectrometry encompasses a broad range of sampling and ionization techniques. To date, only a small subset of these, based on liquid microjunction extraction, have proven suitable for intact protein analysis from thin tissue sections. Liquid extraction surface analysis shows particular promise for this application. Recently, a range of ion mobility spectrometry approaches have been coupled with ambient mass spectrometry. Improvements in signal-to-noise ratios, decreased chemical noise and separation of molecular classes have been described for the analysis of various biological substrates. Similar benefits have been described for ambient mass spectrometry imaging studies. In this review, we discuss the application of ambient mass spectrometry and ion mobility spectrometry to the analysis of intact proteins, and discuss opportunities and challenges for the field.

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**Current Opinion in Chemical Biology** 2018, **42**:67–75

This review comes from a themed issue on **Omics**

Edited by **Erin Baker** and **Perdita Barran**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 16th January 2018

<https://doi.org/10.1016/j.cbpa.2017.11.002>

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## Introduction

Ambient ionization (AI) mass spectrometry (MS) refers to a range of techniques which enable the direct analysis of sample surfaces under ambient conditions [1]. Ambient ionization mass spectrometry offers a number of benefits for intact proteins over the traditional alternative of matrix-assisted laser desorption/ionization (MALDI). These include minimal (or no) sample preparation (simultaneously eliminating low  $m/z$  interference from MALDI matrix ions), the opportunity to couple to high resolution mass analysers owing to the generation of multiply charged ions (MALDI analysis of intact proteins is typically coupled to ToF mass analysers to enable detection of singly charged protein ions at high  $m/z$ ) and the opportunity to rapidly analyse multiple molecular classes simultaneously by careful selection of sampling solvent.

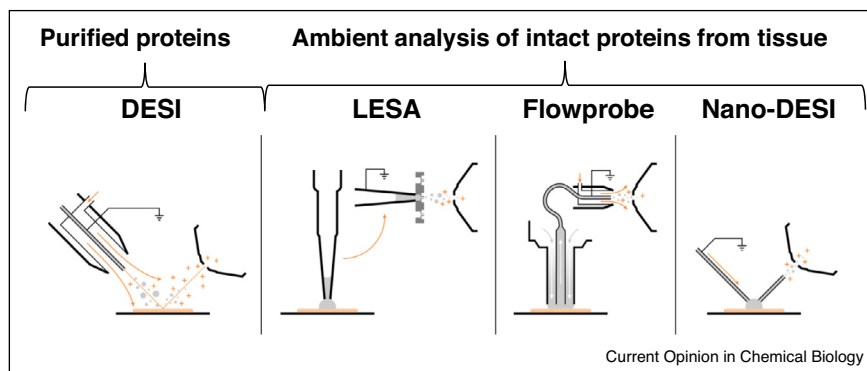
The ability to probe solid substrates directly offers several advantages over analysis of liquid samples; most obviously, the opportunity to access spatial information (e.g., to perform mass spectrometry imaging). For clinical samples, solid substrates may present a safer option than liquid samples: In the case of dried blood spots, for example, infections such as HIV cannot survive the drying process [2], thus they are safer than whole blood.

This review focuses on the application of ambient ionization mass spectrometry for the analysis of intact proteins. Each technique is described alongside reported applications with respect to analytes detected and surfaces probed. A recent development has been the hyphenation of ambient ionization mass spectrometry with ion mobility spectrometry, including high field asymmetric waveform ion mobility spectrometry (FAIMS), drift tube ion mobility spectrometry (DT-IMS) and travelling wave ion mobility spectrometry (TWIMS). These developments, and specifically the benefits for protein analysis, will be discussed. Lastly, the incorporation of ion mobility spectrometry into ambient mass spectrometry imaging workflows will be considered.

## Ambient surface mass spectrometry

In recent years there have been significant developments in the number of AI techniques available for surface sampling [3,4]. Perhaps most well-established is desorption electrospray ionization (DESI), which desorbs analytes from surfaces using a jet of charged solvent ions, and which has been applied to a wide range of samples. DESI has been described for the analysis of drugs [5,6], lipids [7,8] and proteins [9] from substrates including tissue [5,7,10,11,12], dried blood spots [6], bacterial colonies [13] and pharmaceutical tablets. Applications including the profiling of biomarkers in carcinoma tissues [7,10], assessment of drug distributions [5,14], quantitation of drugs in dried blood spots [6] and monitoring of bacterial [13] and fungal [15] metabolism have been described. Whilst DESI provides a rapid AI technique for the analysis (and imaging) of small molecules, the analysis of much larger (protein) species from solid substrates is limited. To date, only certain protein standards spotted onto surfaces have been successfully detected, and limits of detection increase exponentially with protein mass [16,17]. Although this limitation was originally believed to be due to increasing difficulty in physical desorption of the protein from the surface, Douglass *et al.*, showed that the issue was incomplete dissolution of the protein during

Figure 1



Schematics of ambient ionization mass spectrometry techniques (a) desorption electrospray ionization (DESI), (b) liquid extraction surface analysis (LESA), (c) flowprobe sampling and (d) nano-DESI.

desorption [17]. In their conclusion, they note that liquid microjunction sampling techniques circumvent this problem as the dissolution and ionization processes are separated in time. Recent reports describe the use of solvent additives to improve signal-to-noise ratios in DESI of purified proteins [18<sup>\*</sup>]. Haemoglobin has been detected from lysed cells [9] by DESI MS, however DESI of proteins directly from thin tissue sections has not been described.

As suggested above, analysis of intact proteins by ambient mass spectrometry is largely confined to liquid microjunction surface-sampling (LMJ-SS) coupled to electrospray ionization (ESI). Numerous variations on LMJ-SS exist [4], however the following techniques dominate the literature; liquid extraction surface analysis (LESA), Flowprobe sampling and nano-DESI. In LESA [19], the substrate is sampled with a predetermined discrete volume of solvent (2–10  $\mu\text{l}$ ) by use of an Advion Nanomate<sup>TM</sup> robot. Sampling is followed by nano-ESI; the two events are decoupled but automated. Conversely, Flowprobe sampling involves continuous solvent delivery to the surface through an outer capillary (typically  $\sim 40$ – $100 \mu\text{l min}^{-1}$ ) before aspiration away from the surface through an inner capillary [20], and ionization by ESI. Despite its name, nano-DESI is not a variant of DESI but makes use of continuous flow liquid microjunction sampling in a similar manner to Flowprobe sampling, however, the solvent flows between two separate capillaries via a solvent bridge [21]. Figure 1 shows schematics of DESI and the three liquid microjunction surface sampling techniques.

Similarly to DESI, LESA MS has been applied to the analysis of a wide range of (non-protein) analytes including chemical residues on food [22], peptides (digested proteins) from meat products [23,24] and biomaterial surfaces [25], lipids from tissue samples [26–28], single

cells [29] and worn contact lenses [30], drugs from dosed animal organs [31–34], organic compounds from aerosols [35], metabolites from fungi [36] and skin blanching compounds [37]. Unlike DESI, however, LESA has also been widely applied to intact protein analysis from substrates including dried blood spots on card [38–40], bacterial colonies grown on agar [41,42] and thin tissue sections on glass slides [43]. Recently, LESA has even been shown to be compatible with the extraction of non-covalent protein complexes (tetrameric haemoglobin) from dried blood spots [44] and tissue [45<sup>\*</sup>,46] by use of native-like extraction/ionization solvents. Moreover, by use of micelle-containing solvents, LESA of purified membrane proteins on glass substrates has been demonstrated [47]. Proteins of up to  $\sim 40$  kDa have been extracted from tissue sections by LESA MS on an orbitrap mass spectrometer [48<sup>\*\*</sup>] and up to 64 kDa on a Q-TOF mass spectrometer [45<sup>\*</sup>]. For purified protein standards, the current record for molecular weight analysed by LESA MS is  $\sim 800$  kDa [47], recorded on a Q-TOF mass spectrometer.

An additional advantage of LESA sampling, again due to the opportunity for decoupling extraction from ionization, is that extracted samples can be manipulated prior to ESI. Recently, a ‘bottom-up’ LESA proteomics approach, in which automated trypsin digestion of proteins extracted from dried blood spots and thin tissue sections by LESA followed by liquid chromatography (LC) tandem mass spectrometry of the resulting peptide mixture, was demonstrated [49,50]. Similar protocols in which on-tissue digestion is followed by LESA sampling of tryptic peptides have been described [51]. Extracted peptides were either spotted onto MALDI targets or separated by LC-MS leading to the identification of large numbers of proteins/peptides [51,52]. In that work, the opportunity to perform proteomics experiments on fixed tissue samples was demonstrated.

For both Flowprobe MS and nano-DESI, the majority of applications have focussed on non-protein analytes; however, reports of protein analysis are just starting to emerge. Flowprobe MS has been described for the analysis of metabolites from a range of microorganisms [53,54], lipids [55] and drugs [56] from TB-infected lung tissue, drugs from dried blood spots [57] and even for the analysis of dyes in prehistoric textiles [58]. Protein analysis directly from tissue samples has recently been described from murine brain [59] and human ovarian cancer tissue [60]. In our work [59], we found that the sensitivity of Flowprobe MS for proteins was lower than that observed with LESA MS. Nano-DESI has been applied to the analysis of lipids and metabolites in living bacteria [61,62] and tissue samples [61,63–66], drug monitoring from skin sample pads [67], dried blood spots [68], drug-dosed animal organs [69] and pharmaceutical tablets [70], organic compounds in fuels [71,72], air samples [73] and aerosols [74–76], neurotransmitters [77] in tissue samples and even lipids in embryos to better understand premature birth [78]. Hsu *et al.* recently described nano-DESI imaging of proteins of molecular weight up to 15 kDa in murine brain [12\*].

### Coupling of ambient surface mass spectrometry and ion mobility spectrometry

An inherent challenge for ambient surface mass spectrometry, particularly of biological substrates, is that of sample complexity. Typically in mass spectrometry experiments, the challenge of sample complexity is met by use of liquid chromatography, either on-line or for sample prefractionation. The only ambient mass spectrometry technique compatible with that approach is LESA as the extraction and ionization processes can be completely decoupled. Nevertheless, the use of LC in a LESA workflow counters the benefit of rapid analysis, and is especially undesirable in mass spectrometry imaging experiments. An alternative approach is fast (millisecond timescale) gas-phase separation by coupling of ion mobility spectrometry. One such technique is high-field asymmetric waveform ion mobility spectrometry (FAIMS, also known as differential mobility spectrometry (DMS)) [79], in which ions are separated at atmospheric pressure on the basis of differences in their mobility in high and low electric fields. (It should be noted that as separation occurs at atmospheric pressure, FAIMS is not compatible with standard (vacuum) MALDI.) Initial work in this area coupled FAIMS with ambient surface mass spectrometry for the analysis of small molecules. Fernandez and co-workers coupled FAIMS with DESI for the analysis of counterfeit pharmaceuticals [80] and the imaging of lipids in brain tissue [81]. FAIMS has also been shown to reduce chemical noise in DESI oil analysis [82]. Porta *et al.* coupled FAIMS with LESA MS for the analysis of drugs of abuse and their metabolites, demonstrating improvements in signal-to-noise [83].

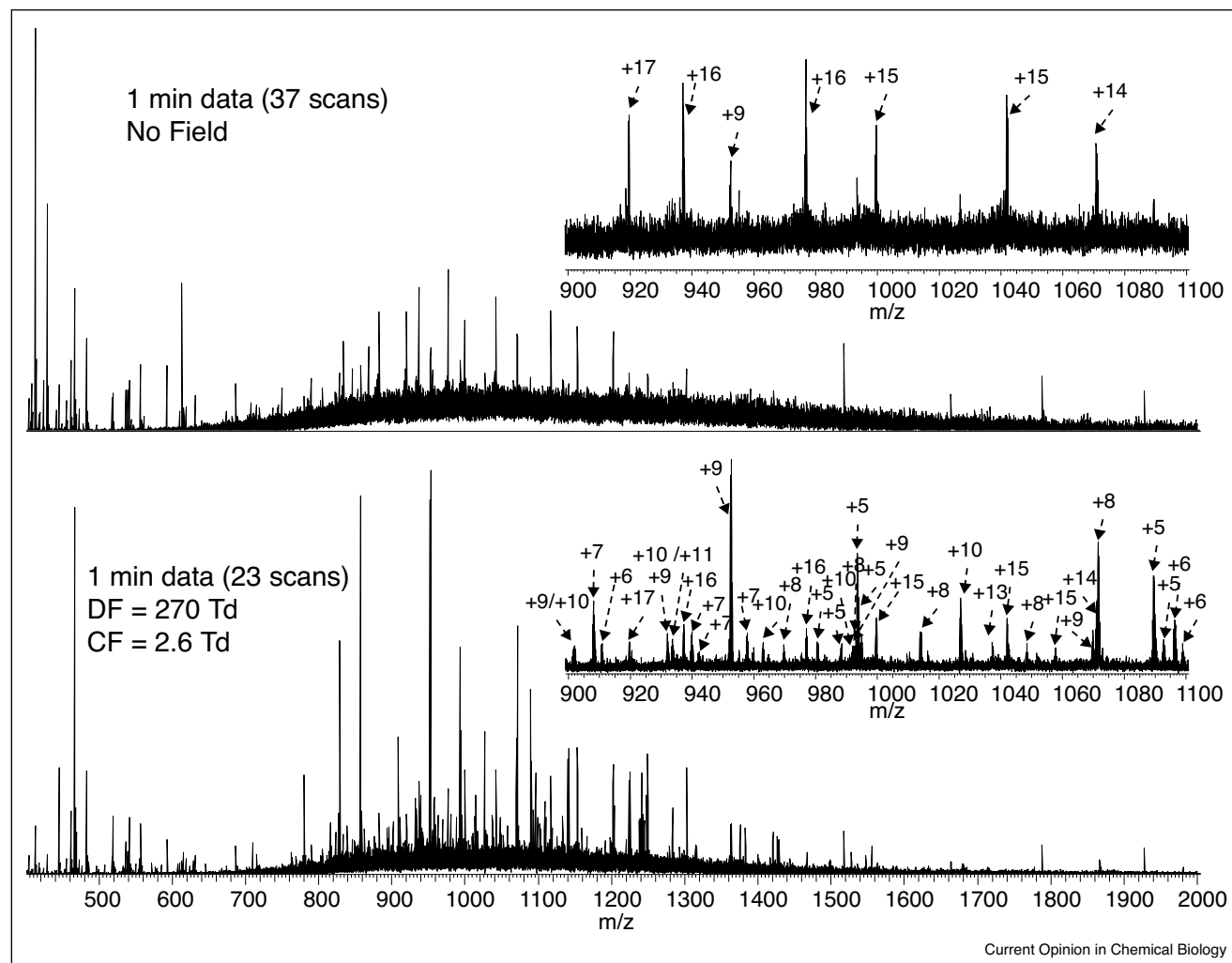
Recently, the integration of FAIMS with LESA MS was described for the analysis of proteins directly from biological substrates [48\*\*]. The benefits include improved S/N, reduced chemical noise, shorter analysis times, and separation of molecular classes. LESA FAIMS MS of proteins has been demonstrated for dried blood spots, bacterial colonies growing on agar and thin tissue sections [48\*\*,84\*\*,85\*]. Figure 2 shows the particular improvements in protein detection from thin tissue sections of mouse brain afforded by FAIMS separation. An associated benefit of LESA FAIMS MS of biological substrates is the ability to filter out abundant proteins, for example, LESA FAIMS MS of dried blood spots enables the monitoring of singly charged lipids that are otherwise masked by abundant haemoglobin signals [85\*]. This advantage is illustrated in Figure 3.

Other ion mobility spectrometry techniques have also been coupled with ambient surface mass spectrometry. Drift tube ion mobility spectrometry (DT-IMS) has been coupled with DESI for the analysis of drugs from TLC plates [86] and pharmaceutical formulations [87,88]. Travelling wave ion mobility spectrometry (TWIMS) has been coupled with DESI for the reduction of spectral complexity and simplification of data analysis in the imaging of multiply-charged gangliosides from murine brain tissue [89] and pharmaceutical tablets and preparations [90]. To date, however, there are few publications which describe the application of TWIMS coupled to ambient mass spectrometry for the analysis of intact proteins. Clemmer and co-workers combined DESI and TWIMS for the analysis of pure samples of cytochrome c and lysozyme. Our group has combined LESA and TWIMS for the analysis of proteins in thin tissue sections of mouse brain [46]. Unlike FAIMS, TWIMS may be used to calculate collision cross sections (CCS) of ions, achieved by comparison to a calibration of known CCS values of protein standards. We showed that it is possible to calculate the CCS of a range of protein ions including ubiquitin,  $\beta$  thymosin 4 and  $\beta$  thymosin 10 extracted from brain tissue by LESA TWIMS MS.

### Ion mobility mass spectrometry imaging

A key application of ambient ionization mass spectrometry is in mass spectrometry imaging, and incorporation of ion mobility spectrometry has been reported for a range of MS imaging modalities. Typically, FAIMS is used as an ion filter under specific conditions to improve the S/N of a desired molecular class. For example, FAIMS improved the contrast between on-tissue lipids and off-tissue signals by significantly reducing background chemical noise in DESI imaging of mouse brain tissue sections [91]. As described above, however, DESI has limited applications in the analysis of larger molecules such as proteins and has not been applied to imaging of proteins in tissue. The first report of ambient mass spectrometry imaging coupled

Figure 2



LESA FAIMS mass spectrometry of mouse brain: static FAIMS mode. (Top) LESA mass spectrum obtained in the absence of FAIMS field. (Bottom) LESA FAIMS mass spectrum obtained at DF = 270 Td, CF = 2.6 Td. Inset: Expanded  $m/z$  regions. Both mass spectra comprise 1 min of data.

Source: Reproduced from Ref. [48\*\*]. Published by the American Chemical Society.

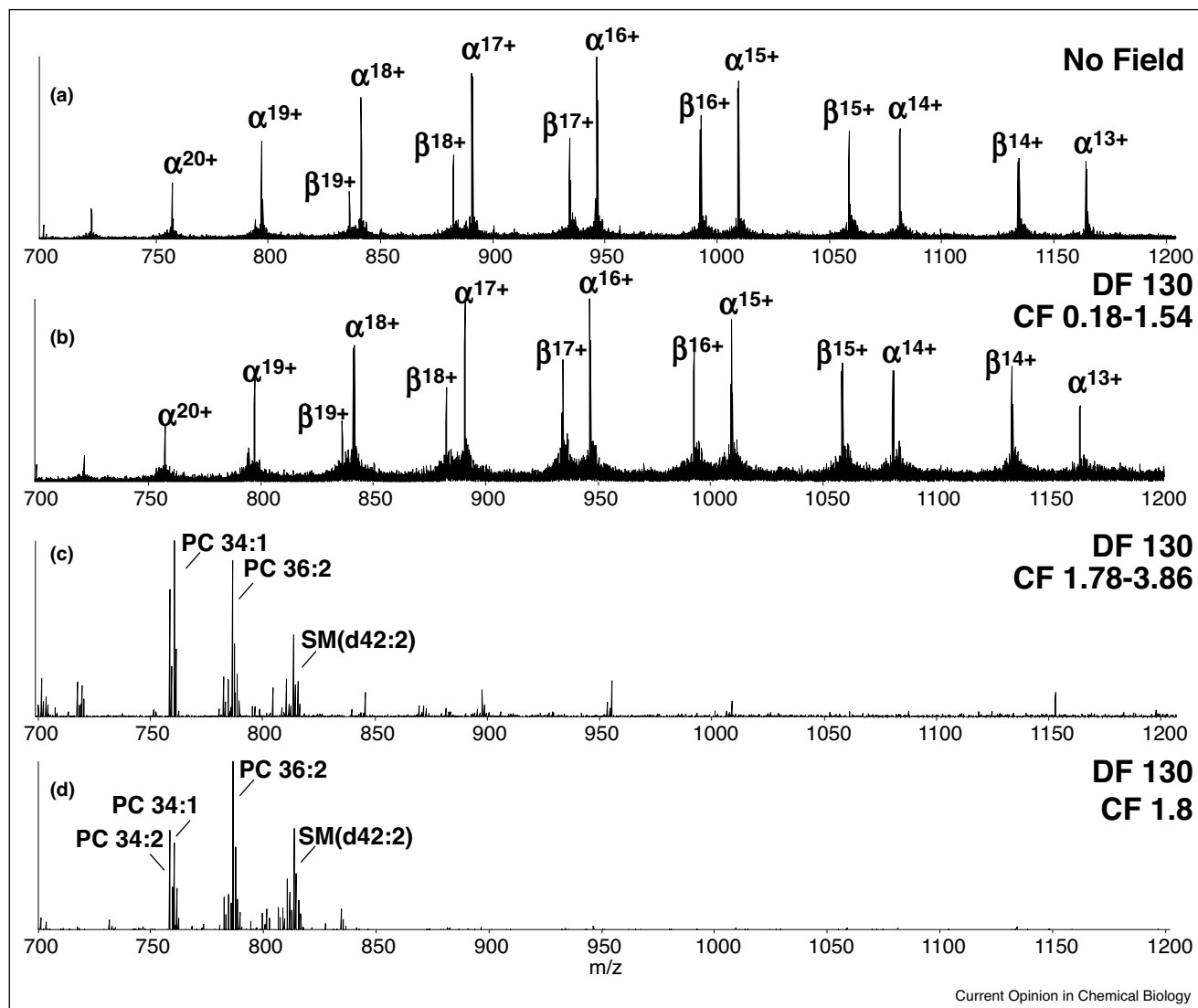
with ion mobility spectrometry of proteins described the hyphenation of LESA mass spectrometry imaging with FAIMS [84\*\*]. Figure 4 shows LESA FAIMS ion images obtained from mouse liver. When compared with LESA mass spectrometry imaging in the absence of FAIMS, a wider range of intact protein species were detected owing to reduced chemical noise combined with improved signal-to-noise ratios. The greatest limitation to ambient protein imaging via LESA is spatial resolution, or more precisely achievable pixel size (1–2 mm). That can be slightly improved by use of continuous-flow sampling via the Flowprobe system: the liquid-microjunction size can be confined to the size of the probe tip (600  $\mu\text{m}$ ) owing to finer controls of the probe to sample height, however is offset by reduced sensitivity. Similar benefits of FAIMS were reported via Flowprobe MSI (630  $\mu\text{m}$  resolution) of

intact proteins from murine brain and human ovarian cancer tissue [60].

### Conclusions and future directions

Ambient ionization mass spectrometry is emerging as a useful tool for intact protein analysis. The primary tool for this application to date has been liquid extraction surface analysis (LESA). Unlike DESI, LESA enables complete dissolution of proteins and offers good sensitivity. LESA of proteins up to 800 kDa has been demonstrated suggesting there is little, if any, limitation on molecular weight providing the protein is soluble in the extraction solvent. Nevertheless, for tissue samples the current limit for molecular weight is ~64 kDa recorded on a Q-TOF instrument and ~40 kDa on an orbitrap instrument. Future work is likely to focus on bridging

Figure 3



(a) LESA mass spectrum obtained from a dried blood spot (DBS) in the absence of FAIMS; (b) LESA FAIMS mass spectrum obtained from a DBS (2D FAIMS: DF = 130 Td, CF = 0.18–1.54 Td); (c) LESA FAIMS mass spectrum obtained from a DBS (2D FAIMS: DF = 130 Td, CF = 1.78–3.86 Td); (d) LESA FAIMS mass spectrum obtained from a DBS (static FAIMS: DF = 130 Td, CF = 1.8 Td).

Source: Reproduced from Ref. [85]. Published by The Royal Society of Chemistry.

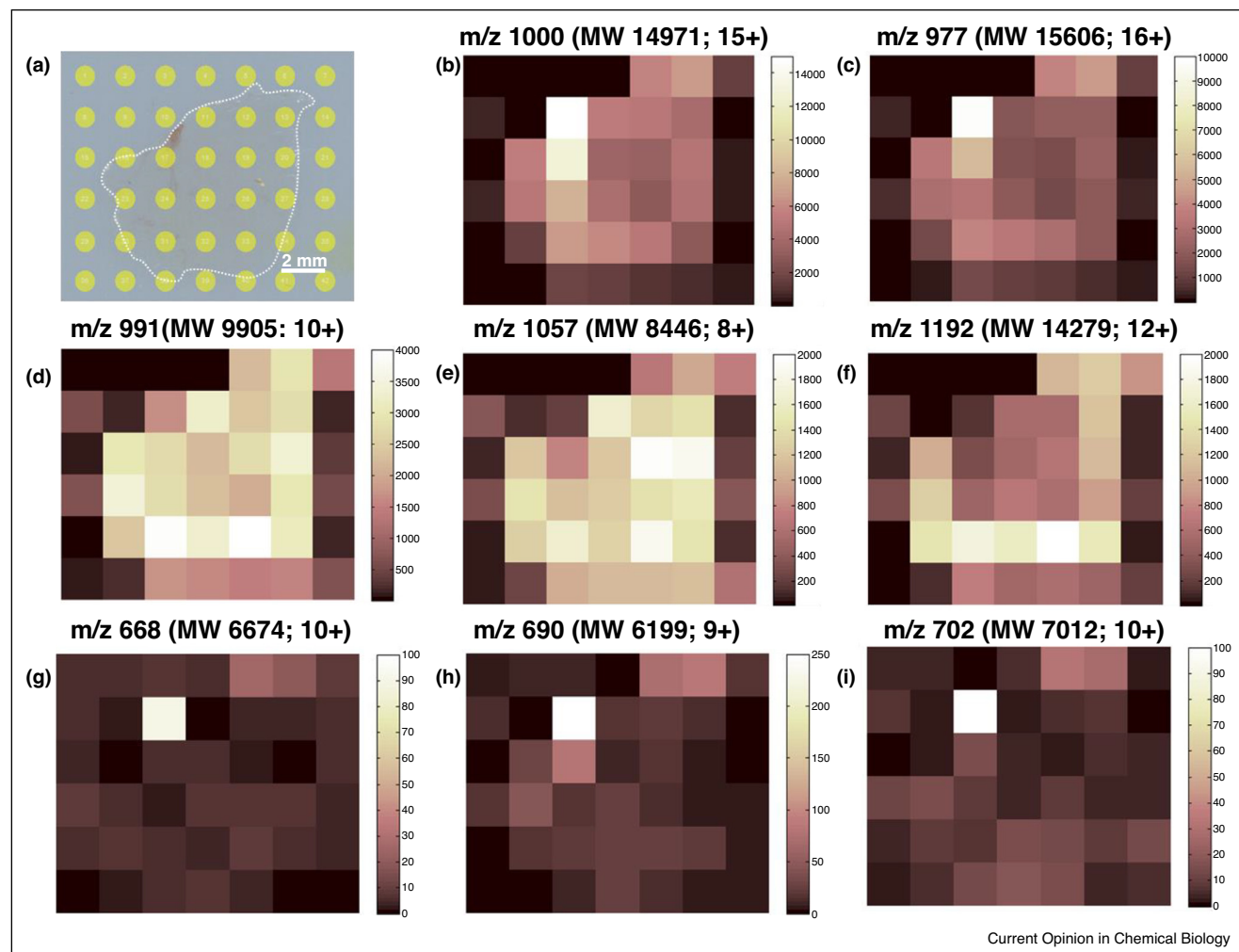
that gap so that high molecular weight proteins can be detected directly from tissue. The incorporation of ion mobility spectrometry is likely to have a significant role in achieving that aim. Research to date has clearly demonstrated the benefits of FAIMS for LESA analysis of proteins through improved S/N and reduced chemical noise. A limitation of LESA is achievable pixel size, which is large (1 mm) in comparison to that achievable via MALDI (typically  $\sim 50 \mu\text{m}$  lateral resolution); nevertheless this can be addressed for ambient protein imaging to some extent by other liquid microjunction sampling techniques such as Flowprobe MS or nano-DESI. The corollary of using these approaches, however,

is reduced sensitivity and limited molecular weight range.

Another exciting possibility for protein analysis is the hyphenation of ambient ionization mass spectrometry with travelling wave ion mobility spectrometry. LESA mass spectrometry of non-covalent protein complexes from dried blood spots and thin tissue sections has been demonstrated. (This capability offers a unique advantage over MALDI which does not provide opportunities for interrogation of non-covalent interactions.) Consequently, there are great opportunities for subsequent structural characterisation, that is, measurement of



Figure 4



LESA FAIMS MSI of mouse liver tissue at DF = 270 Td, CF = 2.68 Td. **(a)** LESA sampling positions, **(b)**  $m/z$  1000 (MW 14 971 Da; 15+;  $\alpha$ -globin), **(c)**  $m/z$  977 (MW 15 606 Da; 16+;  $\beta$ -globin), **(d)**  $m/z$  991 (MW 9905 Da; 10+), **(e)**  $m/z$  1057 (MW 8446 Da; 7+), **(f)**  $m/z$  1192 (MW 14 279 Da; 12+; FABP1), **(g)**  $m/z$  668 (MW 6674 Da; 10+), **(h)**  $m/z$  690 (MW 6199 Da; 9+), and **(i)**  $m/z$  702 (MW 7012 Da; 10+).

Source: Reproduced from Ref. [84\*\*]. Published by the American Chemical Society.

collision cross sections of folded protein ions directly from tissue, to be realised.

## Acknowledgements

HJC and RLG are funded by the EPSRC (EP/L023490/1). KIK is in receipt of an EPSRC studentship in collaboration with the National Physical Laboratory.

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